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The impact of pollen tube growth on stigma lobe curvature in *Kosteletzkya virginica*: The best of both worlds

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Abstract

Kosteletzkya virginica (Malvaceae) is an herbaceous species native to North America that was introduced into China in 1993. Flowers are viable for one day and contain five independent lobes with curving stigmas that are pollinated either by insects or by stylar movement. If pollinated, stigma lobes remain erect, but if pollination fails, stigmas curve towards the anther, eventually making contact and self-fertilizing. We measured 1) the effect of pollination on stigma lobe curvature, 2) the effect of self and outcrossed pollen on pollen tube growth, and 3) the fertility of flowers in which the number of pollinated stigmas varied. Results indicate that the most important signal to halt stigma curvature occurs when the pollen tube grows beyond the stigma lobe. This response was not influenced by the amount of pollen received. The stigma lobes ($n=121$) halted curvature after receiving from 1 to 162 pollen grains. Experimental pollination from one to four stigmas did not inhibit curvature of adjacent un-pollinated lobes. Under open-pollination conditions, the flowers with five stigmas produced significantly more seeds than flowers with one to four stigmas ($U=61.00$, $W=881.00$, $P<0.001$). These results not only provide an experimental test that curvature of individual lobes is independent, it represents the first demonstration that pollen tube growth rather than pollen quantity is the signal that halts stigma lobe curvature. The ability to independently regulate curvature in each lobe allows the plant to produce a mixture of outcrossed and selfed seeds, which can maximize fitness benefits and may represent a "best of both worlds" mating system.

Keywords: *Kosteletzkya virginica*; Pollen tube growth; Pollination; Stigma lobe curvature

1. Introduction

In many angiosperms, floral structures can undergo dramatic movements over relatively short periods and can influence plant pollination and mating systems. Examples include: (1) stamen (pollen) movement, such as pollen sliding (Wang et al., 2004), pollinia reconfiguration in orchids and asclepiads (Darwin, 1862; Catling, 1990; Peter and Johnson, 2006), anther rotation (Liu et al., 2006), stamen curvature (Rathcke and Real, 1993) and stamen elongation (Eckert and Schaefer, 1998); (2) pistil (style) movement, such as stigma closure (Fetscher and Kohn, 1999; Yang et al., 2004), stigma flexibility (Yu and Huang, 2006), flexistyly (Li et al., 2001) and style curvature (Buttrose et al., 1977; Culley, 2002; Ramsey et al., 2003); and (3) other floral activities such as corolla abscission (Dole, 1990), corolla wilting (Sun et al., 2005), corolla closure (Ruan et al., 2005a), and floral (Huang et al., 2002) or ovarian (Xu et al., 1997) stalk curvature. These distinct floral behaviors may have different adaptive significance, such as reduction in intrafloral male–female interference (Yang et al., 2004; Edwards et al., 2005; Bai and Zhang, 2005), avoidance of self-pollination and promotion of outcrossing (Schlessman, 1986; Li et al., 2001; Verma et al., 2004; Peter and Johnson, 2006), delayed auto-pollination (Wang et al., 2004; Ruan et al., 2004; Liu et al., 2006), and tolerance of harsh environments (Huang et al., 2002; Bynum and Smith, 2001).

Reproductive assurance is one of the most longstanding and widely accepted explanations for the evolution of selfing (Jarne and Charlesworth, 1993; Barrett, 1998; Herlihy and Eckert, 2002). This theory predicts that self-pollination is advantageous in environments or conditions where mates or pollinators are

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rare. Examples of selfing leading to reproductive assurance have been reported in several species (Donnelly et al., 1998; Nagy et al., 1999; Anderson et al., 2003; Elle and Carney, 2003). More comprehensive analyses were conducted by Kalisz et al. (2004) and Herlihy and Eckert (2002) to test population and time effects, and alternatives to the reproductive assurance hypothesis, such as pollen discounting (*sensu* Holsinger, 1996), seed discounting (*sensu* Lloyd, 1992), selfing rates, and inbreeding depression. Kalisz et al. (2004) showed that delayed selfing of *Collinsia verna* supports reproductive assurance. Herlihy and Eckert's research (2002) indicated that auto-pollination in *Aquilegia canadensis* increases seed production; however, this benefit was out-weighed by the loss of high quality seed as a result of seed discounting and inbreeding depression. The above examples were conducted at the level of populations, there has not yet been addressed at the level of individuals or flowers (Zhang, 2004).

The ability to self-reproduce by curving un-pollinated styles towards the anther is intriguing, and is known to occur in several species, including *Hibiscus laevis* (Klips and Snow, 1997), *Hibiscus trionum* (Buttrose et al., 1977), and *Kosteletzkya virginica* (Ruan et al., 2005a). In *H. trionum*, curvature is reversible, with partially curved styles regaining an erect position after receiving pollen. The effect of pollination on *H. trionum* is broadly inhibitory such that application of pollen to a single stigma lobe is sufficient to prevent curvature of adjacent lobes (Buttrose et al., 1977). Such behavior is viewed as a potential mechanism to give outcrossing the highest priority, while retaining the ability to self if outcrossing fails (Buttrose et al., 1977).

In *H. laevis*, various hypotheses have been proposed to explain the adaptive significance of curvature in un-pollinated styles, such as effect of delayed selfing (Blanchard, 1976) and encouragement of outcrossing (Stephens, 1948). Curvature is ostensibly adaptive because it positions stigmas in the flight path of nectar-seeking bees subsequent to a predominantly male phase of anthesis, when the stigmas are held together, projecting forward. It remains unclear whether the facultative style curvature in *H. laevis* has a significant effect on the breeding system. For example, in plants from northern populations, curvature of styles results in auto-pollination, but the distance between anthers and stigmas is too great to allow selfing in plants from south populations (Klips and Snow, 1997). It was proposed but not tested experimentally, that the mechanisms underlying reduced curvature of pollinated styles is a function of pollen tube growth, which stiffens the style, thus rendering curvature difficult (Klips and Snow, 1997).

K. virginica (Malvaceae) is an herbaceous perennial native to eastern salt marshes in North America (Gallagher, 1985). Because of its salinity tolerance, it was introduced into China in 1993 to improve soil conditions and provide a sustainable agriculture crop in saline conditions. The fruit capsule contains five ventricles, each with a single ovule. Flowers of *K. virginica* have a monadelphous androecium (filaments are united to form one set or tube) bearing 20–30 anthers surrounding an exerted penta-lobed stigma with synstylous base (Ruan et al., 2005b). Pollination of *K. virginica* occurs through insect pollinators such as *Bombus speciosus* Smith, *Pieris rapae* L., and *Colias hyale* L., as well as delayed self-pollination by stylar movements (Ruan et al., 2005b). Each flower is receptive for one day. If newly-opened flowers are pollinated, stigma lobes remain erect. But if pollination fails to occur, the five-lobed stigmas curve downward, towards and eventually making contact with, the anthers (Ruan et al., 2005a). Previous observations indicate that stigmas cease curving if pollination occurs.

In this paper, we first describe the effect of pollen quantity on curvature of a single stigma and test if the signal for cessation of curvature is pollen tube growth. We also present data on the relationship between pollen quantity, pollen tube growth, and cessation of stigma curvature. Secondly, we test if the application of pollen to individual stigmas in *K. virginica* is sufficient to prevent curvature of adjacent lobes, and we investigate how pollination of varying stigma numbers affects curvature of adjacent un-pollinated lobes. Thirdly, we quantify the fertility of fruits produced by flowers with varying stigma numbers to determine the contribution of selfing by curving styles to seed set. Finally, we discuss the importance of this information to understanding the functional significance of floral traits, and the insight it can provide into the evolution of mating systems in plants.

2. Materials and methods

Seeds of *K. virginica* from the Halophyte Biotechnology Center (University of Delaware, USA) were planted in the Dafeng site for controlled experiments and assessment in 1993. The Dafeng site is located on coastal marsh at Yancheng City, Jiangsu, China. The population density of Dafeng naturalized *K. virginica* was approximately 100,000 in 2005, and approximately 20,000 seeds collected from the site in 2004 were sown in the Dalian site in spring 2005. The Dalian site is located in the coastal wetlands of Dalian city, Liaoning, China. Dalian has a maritime climate with the annual mean temperature of 10.2 °C, and annual mean rainfall of 658.7 mm. It has 191 frost-free days and a total annual radiation of 2764 h.

To observe the effects of pollen quantity on curvature of a single stigma, we applied different amounts of pollen to one stigma lobe per plant in an insect-free greenhouse. We continuously observed a total of 130 pollinated stigma lobes from all treatments to determine if and when lobes ceased curving. The experimental lobes were removed 5 h after pollination, and pollen number estimated using a dissecting microscope (Olympus SZ2-ILST). Portions of treated lobes were observed using a fluorescence microscope (Olympus IX71) to assess pollen germination and tube growth, using the methods of Kalinganire et al. (2000).

We measured the influence of outcross- and self-pollen on stigma lobe curvature. Seventy-five paired selfed and outcrossed stigma lobes from 75 individual flowers were selected, tagged, and observed following pollination. To measure the difference in pollen tube growth rate 160 paired flowers from 160 individuals were collected at 0.5, 1.0, 1.5, 2.0, and 2.5 h after self- and outcross-pollination. Pollen tube growth rates were measured using the fluorescence microscope (Olympus IX71).

To determine the time required for pollen tubes to reach ovules, we used forceps to remove the monadelphous androecium at the

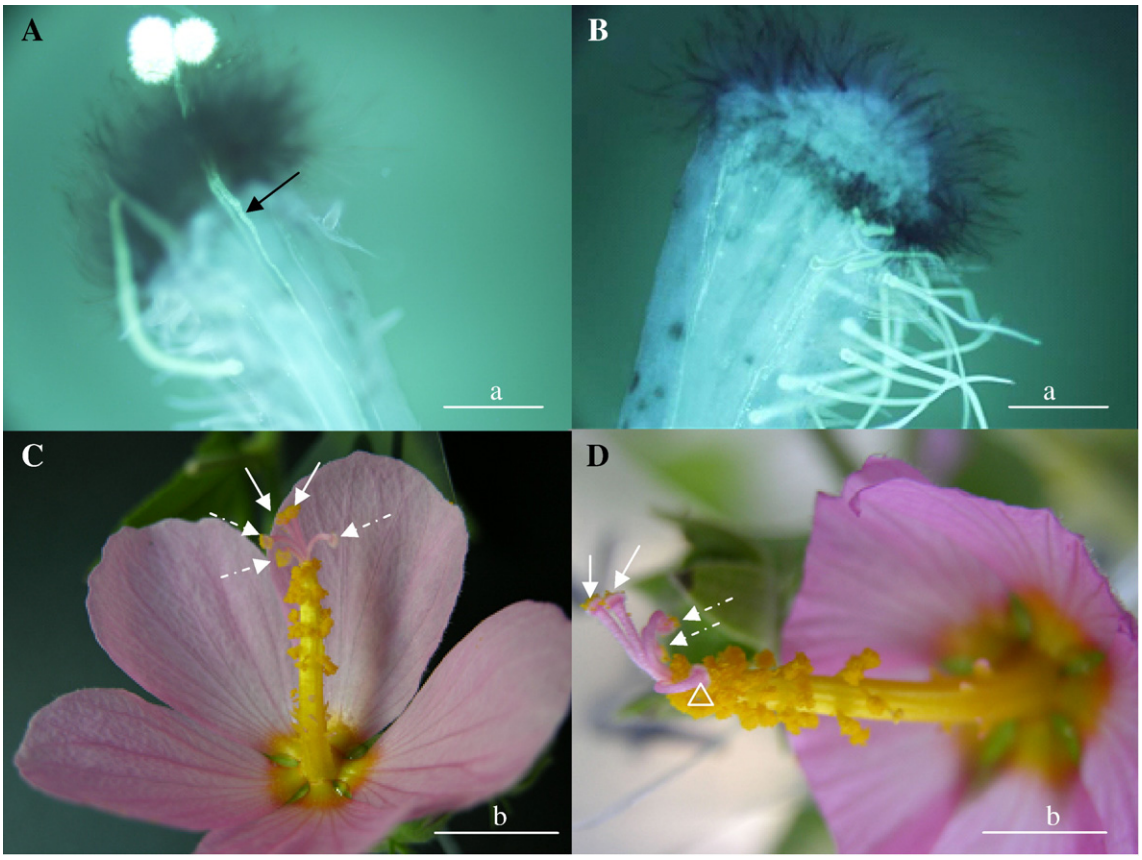

Fig. 1. The relationship between cessation of stigma lobe curvature and pollen tube growth (A and B), and the effects of pollen quantity on the curvature of adjacent un-pollinated stigmal lobes (C and D): (A) After pollination, the stigma lobe ceases to curve downward. Pollen deposited on the stigmatic surface germinated, and the pollen tube (arrowhead) grew beyond the lobe; (B) After pollination, the stigma lobe continued to curve because the pollen tube did not germinate and grow beyond the lobe. Un-germinated pollen is easy to remove by washing the stigma lobe during preparation. C and D showed that the pollination of some of the five stigma lobes did not inhibit curvature of adjacent un-pollinated lobes. In (C) After pollination, two lobes (solid arrowheads) ceased curving. The remaining three un-pollinated three lobes continued to curve downward (dashed arrowheads), but cease immediately upon pollination. In (D) After hand pollination two stigmas (solid arrowhead) ceased curving as remaining un-pollinated lobes continued to curve downward. Two more stigmas were hand-pollinated (dashed arrowheads) and immediately ceased curving; the last stigma (triangle) was not pollinated, and continued to curve until contacting the anthers to self-pollinate. Scale bar: a, 1 mm; b, 1 cm.

junction with the ovary every 30 min for 10 h after hand pollination. If pollen tubes grow beyond the junction and reach the ovule, the flowers with androecia removed can set fruit; in contrast, if pollen tubes do not grow beyond the junction, flowers with androecia removed cannot set fruit.

To observe the effect of pollen quantity curvature of adjacent un-pollinated stigma lobes, different amounts of pollen were applied to one, two, three and four stigmas within a flower. Pollination occurred in an insect-free greenhouse at 08:00. Flowers were continuously observed after pollination to quantify the curvature of pollinated and adjacent un-pollinated lobes. This method is described in Ruan and Jin (2007) and is based on the angle of the stigma lobe relative to the axis of the monadelphous tube. Once pollination occurs, the downward curving motion of the stigmal lobe ceases, and the angle does not change further. In contrast, curvature angle of the un-pollinated lobes continues to decrease gradually. The hand-pollinated lobes in each treated flower were removed 6 h after pollination, and pollen number estimated using a dissecting microscope (Olympus SZ2-ILST). Ten individuals per treatment group were observed each day for 20 days, for a total of 200 flowers examined per treatment.

To measure the fertility of flowers with different stigma numbers, 50 individuals were randomly selected from the Dalian field population. From one to four stigmas were removed from each flower using sharp scissors to produce the following groups: Flowers containing one stigma ($n=10$ individuals with a total of 693 flowers that produced 432 capsules), two stigma ($n=10$ individuals with a total of 816 flowers that produced 505 capsules); three stigmas ($n=10$ individuals with a total of 989 flowers that produced 625 capsules); four stigmas ($n=10$ individuals with a total of 734 flowers that produced 494

Table 1
Effect of pollination of 1–4 stigma lobes on curvature of adjacent un-pollinated stigma lobes

The number of pollinated stigmas	Amount of pollen, mean±1 SE	Observed cessation of curvature in un-pollinated stigma lobes
1	46.11±5.74	No
2	79.49±4.31	No
3	100.45±8.83	No
4	146.92±10.43	No

Note: For treatment of two, three and four stigmas, mean was the average of total pollen grains deposited.

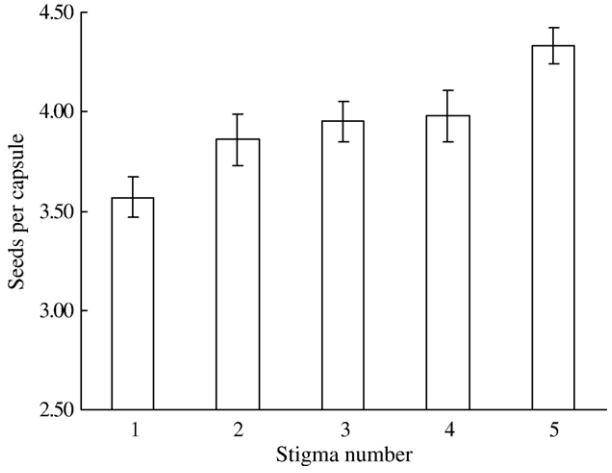

Fig. 2. The number of seed produced by flowers with different numbers of pollinated stigmas. Mean ± 1 SE, $n = 10$ different individuals. Significantly fewer seeds were produced by flowers with one to four stigmas than by flowers with five stigmas ($U = 61.00$, $W = 881.00$, $P < 0.001$, Mann–Whitney U).

capsules); and five stigmas ($n = 10$ individuals with a total of 863 flowers that produced 704 capsules). Mature capsules were harvested to compare the number of seeds produced by flowers with 1–5 intact stigma lobes under open-pollination conditions.

3. Results

Pollination immediately and irreversibly halted curvature in 121 of 130 stigma lobes (mean ± 1 SE = 30.96 ± 0.94 pollen grains, range = 1–162). Pollen tube growth was observed within 1 h after pollination ($n = 35$ lobes, Fig. 1A). Curvature continued in 9 of the 130 pollinated stigma lobes (mean = 18.88 ± 0.63 pollen grains), and no pollen tube growth was observed in this group (Fig. 1B). Both selfed and outcrossed pollen for 75 paired lobes prevented curvature, indicating that pollen source does not influence stigma lobe movement. Thus, under natural conditions, either outcrossing or selfing mediated by pollinators (i.e. geitonogamy and autogamy) can halt stigma lobe curvature. Pollen tubes required 1.0–2.5 h to pass 2.21 ± 0.04 cm style and reach ovules, and there was no difference in growth rate of selfed or outcrossed pollen ($t_{159} = -0.932$, $P = 0.376$).

Pollination of 1–4 stigmas did not inhibit curvature of the adjacent un-pollinated stigma lobes (Fig. 1C and D), regardless of the amount of pollen used (Table 1). This provides an evidence for independent regulation of lobe curvature in each of the five stigma lobes of *K. virginica*.

The number of stigma lobes had a significant effect on the number of seeds produced by flowers in natural pollination conditions ($F_{4, 49} = 6.179$, $P < 0.001$, Fig. 2). Significantly fewer seeds were produced by flowers from one to four stigmas than by flowers with five stigmas ($U = 61.00$, $W = 881.00$, $P < 0.001$).

4. Discussion

Our results are the first confirmation that pollen tube growth, not pollen quantity, is the signal that halts the downward curvature of stigmas towards the central monadelphous androecium. The independence of individual stigmatic lobes in response to pollination and curvature cessation is also a novel discovery. In *K. virginica*, stigma curvature halts when the germinated pollen tube grows beyond the stigma lobe. However, it does not appear to be a relationship between cessation of stigma curvature and the amount of pollen received. Even one pollen grain is sufficient to halt curvature if it germinates and its tube grows beyond the stigma lobe. This finding is consistent with the results of Fetscher and Kohn (1999), that most stigmas in *Mimulus aurantiacus* flowers remain closed permanently once pollen is received, even if the amount is less than needed for full seed set. In contrast, pollinated stigmas in *Campsis radicans* close, but some reopen within 1–3 h, then close permanently if a sufficient amount of pollen (more than 350 grains) is received (Yang et al., 2004).

This study provides experimental evidence that stigma curvature is independently regulated in the five stigma lobes of *K. virginica*. Flowers of *K. virginica* maximize mating opportunities by using multiple pollination mechanisms: insect-pollination and delayed auto-pollination by downward curvature of the stigma lobe. The independent curvature ensures that if outcross-pollination is insufficient to fertilize all ovules, delayed auto-pollination will augment full seed set, which can maximize fitness benefits. In order to demonstrate this, we should consider the following conditions: 1) delayed selfing may not cause pollen and seed discounting; 2) delayed selfing under variable pollinator environments is advantageous even if it lowers progeny fitness; and 3) pollination of a subset of stigmas does not pollinate all seeds in a capsule. All three conditions were validated by our results in this and previous studies.

The flowers of *K. virginica* open fully about 05:00. If stigmas are not pollinated, lobes begin curving around 06:00, and contact anthers at the end of the day, about 14:00–16:00, at which time the corollas close (Ruan et al., 2005a). Several hours of curvature provides enough opportunity for outcrossing. Delayed auto-pollination within a flower occurs after the opportunity for outcrossing has passed, and before the corollas immediately close. Auto-pollination in *K. virginica* not only wastes no pollen, but does not cause seed discounting, because pollen cannot be used by other flowers and the un-pollinated stigmas cannot receive outcross pollen after corolla closure. In hand-pollination treatments for all flowers of 40 plants, each flower set a full compliment of seeds (4.96 ± 0.01 seeds per capsule, Ruan et al., 2004). This showed that delayed auto-pollination in *K. virginica* may not cause seed discounting in other flowers, nor cause a loss of outcrossed seeds for the entire plant.

Delayed auto-pollination in *K. virginica* directly responds to the abiotic environment, as the percentage of flowers displaying delayed auto-pollination was significantly lower on sunny days than on inclement days (Ruan et al., 2005b). This indicates that auto-pollination in *K. virginica* (inbreeding depression was 0.54, Shan et al., 2007) is adaptive for promoting reproductive success under variable pollinators, because emasculated flowers set fewer seeds than intact flowers when open-pollinated (Ruan et al., in press). Fish (1941) showed that selfing is advantageous from the "automatic selection hypothesis;" an allele for selfing will spread

if selfed progeny are at least half as fit as outcrossed progeny (Lloyd, 1979; Nagylaki, 1976). In contrast, Darwin (1876) proposed that an adaptive benefit of self-pollination is to provide reproductive assurance when the opportunity of outcrossing has passed (Baker, 1955; Lloyd, 1992; Kalisz et al., 2004). In addition, self-pollination under variable pollinator environments can be advantageous despite strong inbreeding depression (0.64 for *H. trionum*) (Kalisz and Vogler, 2003; Kalisz et al., 2004; Goodwillie et al., 2005; Seed et al., 2006).

Our fertilization experiments with flowers containing varying numbers of stigmas demonstrate that independent curvature resulting in auto-pollination is advantageous for capsule seed production. In other words, delayed auto-pollination within a flower that has been partially outcross-pollinated provides the potential for fertilizing all ovules. The flowers with one to four stigmas produced significantly fewer seeds than the flowers with five stigmas. After pollination, the stigma lobe halts curvature for the remaining lifetime of the flowers, even if the pollination is not enough for full seed set. Compared to flowers with 1–4 stigmas, flowers with five stigmas have a greater chance for fertilization of the un-pollinated ovule by auto-pollination through stigma lobe curvature. Emasculated flowers (no auto-pollination) produced fewer seeds than intact flowers that were auto-pollinated (2.11 ± 0.05 versus 4.21 ± 0.04, respectively, Ruan et al., in press). The percentage of stigma lobes that were auto-pollination within flowers was 44.61% ± 1.83% (Ruan and Jin, 2007). This mechanism may be similar to stigmas that reopen in *M. aurantiacus* and may be a response to low seed set (Fetscher and Kohn, 1999).

The ability to independently regulate curvature in each lobe allows *K. virginica* to produce a mixture of outcrossed and selfed seeds within fruits, which may represent a "best of both worlds" mating system. Mixed mating system is frequent in seed plants (Schoen and Brown, 1991; Barrett et al., 1996; Johnston, 1998; Vogler and Kalisz, 2001) and often evolves despite strong inbreeding depression (Goodwillie et al., 2005). If the availability of outcross pollen is inconsistent within or between years, such conditions would favor self-pollination (Schoen and Brown, 1991), resulting in a mixture of self and outcross seed production (mixed mating). Unpredictable pollination environments are the norm in wild plant populations, and can include periods of total pollinator failure (Cruden and Lyon, 1989; Burd, 1994). Species with floral developmental mechanisms that promote outcrossing when pollinators are present, but ensure self-pollination if they are not (Lloyd, 1992; Kalisz and Vogler, 2003), can have different annual selfing rates as a functional response to pollinator environments, assuring reproduction and providing a 'best of both worlds' mating system (Cruden and Lyon, 1989). For example, selfing rates of *C. verna* populations directly respond to the pollinator environment, an empirical demonstration for context-dependent benefits to selfing and outcrossing in a variable world (Kalisz et al., 2004). To unequivocally test reproductive assurance under unreliable pollinators, plants must fail to receive outcross pollen, but this failure need not occur every season (Barrett, 2002; Barrett, 2003; Herlihy and Eckert, 2002; Kalisz et al., 2004). In our system, variable weather conditions of coastal tideland result in unpredictable pollinators conditions (Ruan et al., 2005b), and stigma lobe autonomy within flowers varies in response to environmental conditions. For example, the percentage of stigma lobes displaying auto-pollination within flowers was significantly lower on sunny days than on cloudy or rainy days (18.64% ± 1.61% versus 70.58% ± 2.06%, respectively, $U = 0.000$, $W = 465.000$, $P < 0.001$, Ruan and Jin, 2007). This delayed auto-pollination within flowers may provide an opportunity to test the reproductive assurance hypothesis at the level of individuals or flowers, and can provide greater insight into the evolution of mating systems in plants.

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